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NOTCH1 mutations influence survival in chronic lymphocytic leukemia patients

Kerstin Willander1*, Ravi Kumar Dutta2, Jonas Ungerbäck2, Rebeqa Gunnarsson3, Gunnar Julliusson3, Mats Fredriksen2, Mats Linderholm4 and Peter Söderkvist2

Abstract

Background: NOTCH1 PEST domain mutations in chronic lymphocytic leukemia have recently been shown to be of prognostic relevance. Both NOTCH1 and NOTCH2 are constitutively activated in B-cell CLL but not expressed in normal B cells and may be involved in survival and resistance to apoptosis in CLL. We screened for mutations in different parts of both NOTCH1 and NOTCH2 genes and related the changes to survival and other known risk factors.

Methods: In a cohort of 209 CLL patients, we used single strand conformation analysis to determine which of the samples carrying the NOTCH mutations and direct dideoxy sequencing was used to determine the exact nucleotide changes. Kaplan-Meier curves and log rank test were used to determine overall survival for NOTCH1 mutated cases and Cox regression analysis was used to calculate hazardous ratios.

Results: In the present study, we found NOTCH1 PEST domain mutations in 6.7% of the cases. A shorter overall survival was found in patients with NOTCH1 mutations compared to wildtype (p = 0.049). Further, we also examined the extracellular and the heterodimerisation domains of the NOTCH1 gene and the PEST domain and heterodimerisation domain of the NOTCH2 gene, but no mutations were found in these regions. NOTCH1 mutations were most commonly observed in patients with unmutated IGHV gene (10/14), and associated with a more aggressive disease course. In addition, NOTCH1 mutations were almost mutually exclusive with TP53 mutations. In the combined group of NOTCH1 (6.7%) or TP53 (6.2%) mutations, a significant difference in overall survival compared to the wildtype NOTCH1 and TP53 was found (p = 0.002).

Conclusions: Both NOTCH1 and TP53 mutations seem to be independent predictive markers for worse outcome in CLL-patients and this study emphasizes the contention that NOTCH1 mutations is a novel risk marker.

Keywords: Chronic lymphocytic leukemia, NOTCH1 mutations, TP53 mutations, Prognostic markers

Background

Chronic lymphocytic leukemia (CLL) is a heterogeneous disease with variable clinical course characterized by a monoclonal progressive accumulation of mature CD5+ B-lymphocytes avoiding apoptosis. Some patients with an indolent disease need no or little treatment while others have a more adverse disease at diagnosis. No common genetic lesion, which causes the disease, has been found [1], but recurrent mutations in CLL involve TP53 and ATM, and novel mutations in the NOTCH1, SF3B1, MYD88, BIRC3 and FBXW7 genes have been identified through next generation sequencing [2]. The CLL cases may be divided in two major groups regarding to mutated (M) or unmutated (UM) immunoglobulin heavy chain variable region gene (IGHV) where patients with an unmutated IGHV clone have a more adverse prognosis than patients with mutated IGHV gene [3,4]. By the means of FISH analysis, different chromosomal aberrations as deletion in 11q, 13q, 17p or trisomy 12 are found in about 80% of tumor cells in the CLL-patients [5].

Recently, NOTCH1 mutations were found to be predictor of poor prognosis in CLL [6-11]. Furthermore a study of Rosati et al. [12] showed that NOTCH1 and

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NOTCH2, together with their ligands Jagged1 – and 2 are constitutively activated in B-CLL cells but not in normal B cells, suggesting that NOTCH signaling is involved in survival and resistance to apoptosis in CLL.

The NOTCH receptor is a membrane bound protein that consists of an extracellular, transmembrane and intracellular domain that can be released upon ligand interaction and transactivate target genes. The NOTCH signal pathway is activated by a ligand on a neighboring cell and plays an essential role in controlling proliferation, differentiation and survival. Following the receptor-ligand binding, the NOTCH receptor first undergoes a S2 proteolytic cleavage by ADAM proteinase in the extracellular domain, which then is followed by a S3 cleavage by a \( \gamma \)-secretase complex in the transmembrane domain releasing the intracellular NOTCH domain that translocates to the nucleus where it interacts with a transcription complex and acts as a transcriptional activator for multiple target genes [13]. The C-terminal part of the intracellular domain consists of a PEST region that is important for proteasomal degradation of the NOTCH receptor by binding to FBXW7, an E3 ubiquitin ligase, to limit duration of the NOTCH activity. A CT deletion in the C-terminal region results in removal of the PEST domain, a truncated NOTCH protein, and impaired NOTCH degradation and constitutive transcriptional activation of NOTCH target genes in CLL [7,14].

In the present study we have screened for mutations in different parts of both the NOTCH1 and NOTCH2 gene in a cohort of 209 CLL-patients. There is a high structural similarity between NOTCH1 and NOTCH2 genes and recent \( \gamma \)-NOTCH2 gain-of-function mutations are found in B-cell lymphomas [15]. Further, as NOTCH2 is involved in overexpression of CD23, one of the hallmarks of CLL [16], it prompted us to screen both the NOTCH1 and NOTCH2 genes for genetic alterations. Mutations were only found in the PEST region in the NOTCH1 gene in our cohort and emerged as an independent factor of poor overall survival and disease stage, in addition to \( TP53 \) mutations and IGHV gene status.

### Methods

#### Patients

In this study, peripheral blood from 209 CLL patients (145 men and 64 women) was collected between 1996 and 2006 at the Department of Hematology, Linköping University Hospital. Mononuclear cells were isolated by
Ficoll-Paque gradient centrifugation and genomic DNA was extracted by proteinase K digestion and stored frozen until used as earlier described [17]. The samples were collected either at the time of diagnosis or prior to the first treatment. For all patients, follow-up data were available, and for 106 live patients the median follow-up time was 6.8 years (range 1.6-14.9 years). The median age at diagnosis was 62.5 years (range 38.3-87.0 years). The immunophenotype and the Binet staging system were according to the IWCLL guidelines [18]. The immunoglobulin heavy chain variable region genes (IGHV) and TP53 gene status were analysed and reported in an earlier study [17]. Informed consent was obtained from the patients and the study was approved by the regional ethical committee (Dnr 02–459) in Linköping and conducted in accordance with the ethical guidelines of the Helsinki Declaration.

**NOTCH mutation status detection**

The NOTCH1 mutations status were analyzed for the extracellular region (exon 6, 7, 8, 11, 12 and 13), the heterodimerisation domain (exon 26, 27) and the PEST region (exon 34) and the NOTCH2 was only analyzed for mutations in the heterodimerisation and the PEST domains by PCR amplification followed by single strand conformation analysis (SSCA) according to the original protocol [19] and direct dideoxy sequencing to determine the exact nucleotide change and compared to corresponding NOTCH1 and NOTCH2 germline sequence (NM_017617.3 and NM_024408.3 respectively). Primer sequences are shown in Table 1.

**TP53 and IGHV gene status detection**

TP53 gene mutation analysis was performed for exons 5–8 (the DNA binding domains) by the PCR-single strand conformation analysis (SSCA) technique and samples displaying mobility shifts were sequenced with the dideoxy termination method to confirm the nucleotide changes.

The IGHV gene mutational status was performed by PCR amplification on genomic DNA by using specific VH/JH primers [20], followed by DNA sequencing of both forward and reverse strands. To determine the IGHV gene identity the sequences were aligned by using the IMGT/V-QUEST database (http://imgt.org), ≥ 98% identity to the corresponding germline sequence was considered as an unmutated IGHV gene.

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**Table 2 Clinical and biological characteristics of the 209 CLL-patients**

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<td>6</td>
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---

**Figure 1** Kaplan-Meier survival curves displaying a significant difference between patients with NOTCH1 mutation (n = 14) and NOTCH1 wildtype (n = 195) (p = 0.049).

**Figure 2** A significant difference in overall survival between patients with NOTCH1 or TP53 mutations (n = 26) and CLL with non-mutated NOTCH1 and TP53 (n = 183) (p = 0.002).
Statistical analysis
Kaplan-Meier curves were used to show the overall survival and the log-rank test was used to compare the survival between the groups. To calculate hazard ratios (HR) the Cox proportional hazard model (Cox-regression) was used. For all statistical analyses Stata v12.1 was used (StataCorp LP, College Station, TX, USA). P-values less than 0.05 were considered significant. Overall survival was measured from date of diagnosis until the last follow-up or death.

Results and discussion
*NOTCH1* heterozygous mutations in the PEST domain occurred in a frequency of 14 out of 209 patients (6.7%) in our study. Thirteen of the mutations correspond to a 2-bp frameshift deletion, c.7541_7542delCT and one is a novel GT deletion at c.6988_6989delGT, both generating 2-bp frameshift deletion. The frameshift mutations, with subsequent stopcodon and one is a frameshift mutation in our study. Thirteen of the mutations correspond to a frameshift mutation in our study. Thirteen of the mutations correspond to a frameshift mutation in our study.

Table 3 Analysis for overall survival
<table>
<thead>
<tr>
<th></th>
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<th>HR</th>
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<th>P</th>
<th>HR1</th>
<th>95% CI</th>
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<td>1.17-5.53</td>
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</table>
HR, hazard ratio; CI, confidence interval.
1Adjusted for age and sex.

Ten of fourteen cases with mutated *NOTCH1* mutation; this association was not significant (p = 0.56). By univariate analysis, the HR for death increased to 2.27 (1.32-3.91; 95% confidence interval) for tumors mutated in *TP53* compared to *NOTCH1* or *TP53* wildtype tumors (p = 0.003) (Table 3). At the molecular level there seems to be an intriguing and complex link between p53 and *NOTCH1*. P53 induce *NOTCH1* expression and seems to initiate an anti-apoptotic feedback mechanism with subsequent increased cell survival that may limit p53 promoting therapy with e.g. nutlins [28,29]. NOTCH signaling blockade by γ-secretase inhibitors to stimulate apoptosis may be considered to be of therapeutic value at least for wt p53 CLL patients [28].

Among CLL patients with a mutated *NOTCH1* gene 10/14 (71%) had an unmutated IGHV gene in contrast to 113/195 (58%) with a wildtype *NOTCH1* gene, a difference that did not reach statistical significance (p = 0.22).
(Figure 3 and Table 2). CLL with NOTCH1 mutations seemed to be more progressive, with a high frequency of unmutated IGHV gene and advanced Binet stages, indicating a more aggressive disease course.

Five patients in this cohort had NOTCH1 mutation at the time of diagnosis. For these patients the median time to first treatment was 101 days (range 21 to 145 days). For the whole group the median time from diagnosis to the first treatment was 438 days (range 0–6021 days). Further and expected, all patients with NOTCH1 mutations identified at diagnosis had the more advanced Binet stages B and C, a tendency that due to few observations did not reach significance (p = 0.11) (Table 2).

The frequency of NOTCH1 mutations is also reported to be significantly higher in Richter syndrome, i.e., a progression of CLL into diffuse large lymphoma with often dismal outcome [8,30], however our cohort contained no information on the prevalence of Richter syndrome.

It is now recommended to perform TP53 mutation analysis in patients with CLL as TP53 mutations occur in about 5% of cases in absence of 17p deletion and represent an independent prognostic factor associated with worse outcome [31]. CLL patients with 17p deletion and/or TP53 mutations are strongly associated with refractory disease, and also activated NOTCH1 mutations were recently suggested to cause refractoriness to fludarabine [8,9,32].

Conclusions
Our study confirms other recent reports that NOTCH1 mutation eliminating the PEST domain, has a prognostic value as a novel risk marker in CLL similar to TP53 mutations. Thus both NOTCH1 and TP53 mutation may be an indication for earlier and more active treatment or as an indication for transplantation therapy.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
KW collected data, performed experiments, analyzed and interpreted data; JU performed experiments, analyzed and interpreted data; GI interpreted data; MF performed statistical analysis; ML designed experiments; PS designed experiments, wrote the manuscript. All authors were involved in writing the manuscript. All authors read and approved the final manuscript.

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